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Exhaled breath condensate pH assays

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Introduction

Airway pH is central to the physiologic function and cellular biology of the airway. The causes of airway acidification include: 1) hypopharyngeal gastric acid reflux with or without aspiration through the vocal cords; 2) inhalation of acid fog or gas (such as chlorine); 3) intrinsic airway acidification caused by altered airway pH homeostasis in infectious and inflammatory disease processes(1). The recognition that relevant airway pH deviations occur in lung diseases is opening doors to new simple and inexpensive therapies(2). In part, this recognition has resulted from the ability to use exhaled breath condensate (EBC) as a window on airway acid-base balance, about which data are otherwise difficult or ethically impossible to obtain.

EBC consists primarily of water with trapped aerosolized droplets from the airway lining fluid, as well as water-soluble volatile compounds. The pH of EBC is determined primarily by the water-soluble volatile gases(3-5) and reflects (but does not precisely quantitate) airway lining fluid pH. A low pH value in EBC results from the enhanced volatilization of acids that occurs from an acidic source: in other words, an acidic airway lining fluid. Non-volatile basic anions become protonated within an acidic airway lining fluid and thereby turn into volatile (exhalable) acids. Formic and acetic acid(6), as well as numerous other acids, join the exhaled airstream when the airway is acidic, are captured by the water condensing in the exhaled breath, and lower the pH of the EBC. In contrast, when the airway lining fluid is alkaline, the ionized conjugate bases of these acids are the dominant species. The ionized conjugate bases are not volatile, and therefore not appreciably exhaled(5). The association of low airway pH with low EBC pH has been proven empirically with studies of experimental airway acidification in cows(7). These straightforward concepts have allowed a simple assay (pH), performed on easily collected sample (EBC)(8), to provide large amounts of data regarding airway acidity from patients even in the presence of marked acute airway disease(9,10).

Addressing the artifact of carbon dioxide

Most investigators, but not all, address in some manner the important issue of carbon dioxide affecting the EBC pH. CO₂ is a volatile gas that is a precursor to carbonic acid. CO₂ is not appreciably more volatile from acidic airway lining fluid than from alkaline airway lining fluid. Although CO₂ contributes to the acidity of airway lining fluid, the presence of

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Conflict of Interest: JH is a cofounder of Respiratory Research, Inc., which manufactures exhaled breath condensate collection equipment. JH and the University of Virginia have intellectual property interest in airway pH diagnosis and therapy.

CO₂ in EBC does not communicate that the airway lining fluid is acidic, because the CO₂ will be there affecting the pH of the EBC regardless of the airway pH. CO₂ leads to substantial EBC acidification if not accounted for in some fashion and provides noise in the system that limits the ability to identify the smaller effects of the volatile acids, and shrinks the overall effect size of pH measurements in disease by lowering the EBC pH normal range. Gas standardization either with a “CO₂-free gas”(8) or with a gas that contains a known quantity of CO₂ (11) seems wise, and has been commonly adopted.

Gas standardization prior to EBC pH measurement allows for samples to be assayed at any time after collection, and clearly improves assay repeatability and overall reproducibility in patient collections(3,11). Not all CO₂ is removed by gas standardization by means of exposing (bubbling) the sample with a “CO₂-free” gas, in part because it is unlikely that the standardizing gas is truly CO₂-free. Overall, however, the levels of CO₂ in EBC are brought to similar relatively low levels for all samples during gas standardization, but some minor effect of residual CO₂ remains.

The recent work of Horvath's group in Hungary(11) has raised the possibility that using a known concentration of CO₂ for gas standardizing may improve reproducibility of the assay further. There is some loss of effect size between disease and health by so doing, but standardizing on a known concentration of CO₂ may allow for more interlaboratory cooperation. Using “CO₂-free” gas does allow for variable amounts of contaminating CO₂, which—in addition to EBC collection equipment differences—may in part explain why there is some variability of normal in different laboratories.

It should not be a surprise that the volatile acids trapped in EBC are not as affected by gas standardization procedures as the CO₂ is. This is simply because CO₂ is a highly volatile gas that is a precursor of an acid, as opposed to an acid itself, whereas the volatile acids that acidify EBC are trapped by ionization. CO₂ is relatively easy to remove from solution, which likewise removes the carbonic acid that was formed when EBC initially absorbed the CO₂. Thus, the unhelpful effect of exhaled CO₂ on EBC pH is minimized by having the EBC “breathe it back off” during gas standardization.

EBC pH assay validation and technical issues

The gas standardized EBC pH measurements are substantially reproducible and immune to most potential confounding influences. In comparison to other EBC assays, pH is solidly in the range of available assays. Although EBC is a dilute fluid with low ionic strength, there are available pH probes that are designed to function even in distilled water. Thus EBC pH measurement has benefited from the decades of advances in measurement of this standard chemical property. There are readily available technological capabilities to assess concentrations of hydrogen ions as low as 10 femtomolar (which is equivalent to an alkaline pH of 14) and as high as 1 molar (an acid pH of 0). Measurement of pH is routinely performed, even in highly purified water such as that used in steam turbines. Measurement of pH of fluids is a well-understood process with no technical hurdles to overcome.

Our center has specifically examined, and found no effect on the final deaerated EBC pH value of any of the following factors: age (5–75 years), gender or race, time of day collected, volume of EBC collected and duration of EBC collection, degree of hyperventilation or hypoventilation of the subject, temperature or duration of sample storage, choice of CO₂-free gas used for deaeration (oxygen vs. argon), acute use of albuterol/salbutamol by the patient, oral ammonia, or methacholine-induced airway obstruction(3,4,8,12). Note that early data from our laboratory suggest that the very old (> 80 years) reveal more EBC acidification than the rest of the population (unpublished observations).

There do seem to be effects on EBC pH arising from condenser temperature during collection. In healthy subjects (without breath acidification), we found no difference in EBC pH with temperature of collection down to -56°C to room temperature. However, in subjects with acidic breath, collecting at condenser temperatures low enough to cause sample freezing is found to lead to less capture of exhaled acids (which cannot go into solution into ice, after all). Thus we always perform EBC collection at temperatures that do not allow sample freezing during collection.

An additional effect on pH arises during sample storage while frozen. We have noted that after freezing a stored sample with low pH, if the sample container is opened before thawing the measured pH of the sample is higher than the initial measurement. These observations are consistent with sublimation from the solid EBC of relevant volatile acids—a conclusion supported by the ability to prevent this artifact from occurring by allowing the sample to thaw and shaking the container before opening it. Indeed, we strongly recommend this approach.

A final technical comment regarding EBC pH measurement seems warranted. Not all pH probes are appropriate for measurement in the low ionic strength matrix of EBC. Certain technologies simply will not function. We have found that Ross-type pH probes are uniformly effective and standard glass probes with a substantial leak of internal fluid are generally effective. Before we select a probe for our studies, we confirm that it calibrated in low ionic strength buffers identically to normal pH buffers, and that addition of ionic strength adjusters/enhancers to samples of EBC does not affect the pH readings provided by the probe. These two tests help assure that the probe is not adversely functioning in the low ionic strength EBC.

Normal EBC pH values

Normal values of gas-standardized (“CO₂-free”) EBC pH have been reported from multiple investigators and range between 7.5 and 8.1(3,8,9,12-21). In our normative database from 404 subjects, the mean pH was 7.83, and the median pH was 8.0(12). The bell curve data distribution cuts off at approximately pH of 7.4, with a scattering of values below that level that are clearly not part of the normal distribution. EBC pH values below 7.4 occur in 6% of this population. These likely represent subjects with airway acidification that may be transiently symptomatic or asymptomatic, and may result from proximal acid reflux or from temporary innate immune responses against viral infections. From these data and data from over 6000 samples collected from over 600 subjects in other studies, our laboratory considers pH values less than 7.4 to be abnormal.

There are potential confounders of the EBC pH levels. We have found that substantial intake of ethyl alcohol to the point of inebriation may modestly lower EBC pH. This may reflect alcohol's metabolism to acetic acid, which when exhaled can then alter EBC pH. It also may reflect alcohol-induced augmentation of gastroesophageal reflux to the laryngeal level. Additionally, oral ingestion of acidic beverages, and particularly fluids with volatile acids (vinegar) within 20 minutes of EBC collection may cause substantial artifact, with a lower EBC pH. Our protocol for providing EBC sample for pH assay includes that nothing except water should be ingested for 30 minutes prior to collection.

Anatomic source of the determinants of EBC pH

It is reasonable to assume that any significant acidification of the airway lining fluid that is exposed to the exhaled airstream will add acid to EBC. In patients who are endotracheally intubated, pharyngeal contribution will be nil. As part of the airway, the oropharynx likely can contribute to EBC pH when the sample is collected orally(22), but it does not in general

have a strong effect. In keeping with this, salivary pH and EBC pH do not correlate(3), and pH of EBC samples collected by oral breathing is the same as that collected subsequently from the endotracheal tube following intubation in patients undergoing elective surgery(3). That being noted, intentional profound experimental acidification of the pharynx can lead to temporary EBC acidification(13). This reflects the ability of EBC pH assays to identify the presence of a source fluid, at any airway level, sufficiently acidic to protonate certain bases to their volatile conjugate acids (acetic, formic acids).

Although the mouth can contribute to acidity of EBC, it also contributes to alkalinization of EBC by means of ammonia (NH_3) which is a volatile base produced in the mouth and lungs of many people in high concentrations and often is the highest concentration substance measurable in EBC(4). Initial speculation about NH_3 controlling EBC pH was forwarded (23), prompting studies to collect the empiric evidence has allowed for an improved understanding of NH_3 's role. Several interesting observations have been made in regard to oral ammonia. First, removal of potential oral ammonia contribution to EBC by bypassing the mouth by endotracheal intubation does not affect EBC pH. Second, removal of ammonia (likely along with other volatiles) from EBC by lyophilization and resuspension does not affect EBC pH. Third, it is uncommon to have a low EBC pH value without a low NH_3 level in EBC, however it is common to find low NH_3 levels with normal EBC pH levels(4). These observations have lead to our current understanding that high oral production of NH_3 can blunt the EBC pH signal of volatile acids coming from the lungs, and thereby decrease the sensitivity of EBC pH assays to identify lung acidification. However, when the acids exhaled from the airway are sufficient to overcome the modest neutralizing effect of oral NH_3 , a low EBC pH signal represents low airway lining fluid pH. An additional albeit less confident notion we have is that minor differences in EBC pH values between groups within the pH range we consider normal is of uncertain meaning.

Extensive data have now been collected from intubated humans revealing that EBC pH is low in disease states(10). Recently, it has been demonstrated in 12 patients that an endobronchially intubated healthy lung produces an EBC that is alkaline, whereas when the contralateral TB infected lung destined for excision is included in the EBC sampling, the values are acidic(24). These data provide invasive measurements necessary to help convince us that acidification of the lower airways indeed occurs and, as one would expect, indeed is heterogenous—with pH low in the sick portions of the lung.

Research and clinical utility of EBC pH measurements

EBC pH is now perhaps the most commonly performed EBC assay. There are likely two reasons for this. Foremost is that investigators realize that airway pH deviation is a particularly relevant pathologic process that affects most every other aspect of lung disease that may interest them. One cannot ignore such a core component of how the airways and lungs function. Secondly is that the pH assay is relatively easy. Unlike most other assays in EBC, there is no value of pH that is “undetectable”. However, this is misleading. pH measurement represents a net proton concentration signal resulting from amounts and ratios of multiple acids and bases within the EBC. A “normal” EBC pH may mean that there are no volatile acids present (which can therefore be interpreted as “undetectable”). Or it may mean that volatile bases from an alkaline mouth or alkaline proximal airway have neutralized the volatile acids arising from an acidic area in the distal airway (thereby suggesting that EBC pH has relevant limitations to its sensitivity, similar to other EBC assays). A low EBC pH can confidently be said to result from a low airway lining fluid pH at some level. However a normal EBC pH does not exclude that there is airway acidification present, unless there is little NH_3 in the EBC as well.

There are no doubt publications in the literature that have suffered from unrecognized deficiencies of pH probes, effects of freeze-sublimation that artifactually raises EBC pH, and excessively cold condenser temperatures. There are likely more technological validations that remain to be undertaken, but the field has advanced well, and application of this assay as an outcome measure in large clinical studies as well as to assist in clinical patient management are now reasonable considerations.

Continuous EBC pH monitoring in intubated patients

A system has been developed by Respiratory Research, Inc. (a University of Virginia faculty owned company of which the author of the current text is a director) and the United States Air Force that allows for measurement of EBC pH continuously. Known as the ALFA monitor, this system condenses breath from the expiratory port of mechanical ventilators, performs gas standardization at two levels using hospital wall oxygen supplies, and measures, displays and records the EBC pH in digital and graphical formats. Because of the time delay for condensation and processing, the result is a second-to-second recording of an EBC pH moving average representing EBC production from the lungs during the previous 5 to 10 minutes. To date, we have performed continuous pH condensimetry on 40 humans and multiple pigs with various naturally or experimentally-induced lung diseases and find prominent acid production during disease worsening. With the oropharynx eliminated as a source of volatile acids, this system allows for selective measurement of lung acidity. In the absence of oral NH_3 contribution, the EBC pH becomes a higher sensitivity assay for airway acidity.

Diseases for which we have continuous pH condensimetry anecdotal data revealing lung acidification include acute asthma, cystic fibrosis, COPD, infection with respiratory syncytial virus, and ARDS. In these disease processes, pH changes gradually over hours to days. EBC pH > 7.4 is commonly associated with relative lung health, and pH levels with disease fall into the 4–6 range(10), similar to pH found in oral EBC collections from healthy and ill subjects respectively(8). In unpublished data, we have observed frequent gradual decline in EBC pH during general anesthesia, which is as yet of uncertain significance. Additionally, particularly when the endotracheal tube is not cuffed, we have observed rapid pH fluctuations that last for 10–15 minutes that are most likely attributable to gastric acid reflux and aspiration.

This technology is functioning for research to study time course of pH changes in the airways. At this time, knowing that the airway lining fluid pH is abnormally low may be only prognostically valuable for there is no clinically available therapeutic that is approved for use to neutralize acidic airway pH. But airway pH modifying therapies are being tested, and when available will create a desire to know what the airway pH is in a given patient so that these airway pH modifying therapies appropriately can be used.

Assessing for acid reflux-induced respiratory symptoms

Initially, others and we considered that acid reflux could confound EBC pH assays and prevent us from gaining the insights we wanted regarding airway pH. However, with time, we have realized that the airways can acidify intrinsically or extrinsically, with the extrinsic pathways being inhalation of acid gases and aspiration of acid reflux. When considering things that confound, acid reflux is at the top of the list, for it confounds or complicates every respiratory illness. There is perhaps no more diagnostically frustrating entity in pulmonary medicine than acid reflux, because 1) the diagnostics are invasive and not designed or validated for use for respiratory system indications (e.g.—esophageal pH probes, which use normal reference ranges relevant to esophageal disease but irrelevant to aspiration disease); 2) acid reflux is so common.

EBC pH offers a non-invasive assessment of airway pH that can be performed repeatedly. For example, transient acidification of the breath associated with chronic cough appears to be an excellent indicator that the patient's cough will respond to acid blockade therapy with proton pump inhibitors (PPI). In our studies, patients who responded to PPI by decreasing their cough symptoms invariably had transient breath acidification, and those whose cough failed to respond had no breath acidification (Figure 1). These findings provide for a novel method to determine which patients with asthma or COPD have confounding acid reflux without resorting to expensive and indefinite (and commonly indeterminate) PPI empiric trials.

Studies of lung diseases in spontaneously breathing subjects

The initial purpose for which we developed the EBC pH assay was to open a window onto lung chemistry, with pH being certainly a centrally involved characteristic. The results of our work and those of others using the EBC pH assay has provided the strongest support to date that the airways become acidified in numerous respiratory disorders. The EBC pH assay, although not perfect, has undergone validation and vetting internationally. It is our current opinion that the settings in which EBC pH decline is most relevant are in 1) acute asthma exacerbations; 2) COPD ; 3) Acute lung injury and ARDS (representing intrinsic airway acidification), and for 4) assessing gastric acid aspiration in the settings of chronic cough, asthma, COPD, lung transplant rejection, pulmonary fibrosis, vocal cord dysfunction and exercise induced dyspnea/bronchospasm. This purpose may be the most immediately valuable from a patient point of view, for there are therapies to help reflux induced symptoms if a diagnosis is made.

Conclusion

EBC is the only currently available convenient non-invasive ethically acceptable method of assessing airway acidity, especially when it comes to repeated sampling from acutely ill patients. Like all tests, EBC pH is neither perfectly sensitive nor specific for lower airway acidification. Many of the validation issues have been adequately addressed. It remains necessary to interpret the data cautiously, for acid at any level in the airway can lead to EBC acidification, and an absence of EBC acidification does not exclude the presence of some degree of airway acidification. EBC pH measures remain the most successful manner to date to evaluate the role of airway acidification in respiratory disease.

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Figure 1. EBC pH values from patients who respond or do not respond to proton pump inhibition for their chronic cough (a) and non-coughing controls (b). Note that there are multiple samples collected from each patient, with all data plotted as individual samples. Approximately 50% of the EBC samples from PPI-responsive coughers had low pH values. Note that all PPI-responsive patients had at least one episode of breath acidification during 4–8 breath collections collected at the time of cough. Adapted from reference ¹³.